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BY

C. M. HUTCHINSON

Imperial Agricultural Bacteriologist

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INTRODUCTION.

THIS investigation, the results of which are described in this paper, was undertaken at the request of the Assistant Commissioner of Excise for Bengal, Bihar and Orissa and Assam with a view to ascertaining whether the manufacture of *bákhar* can be advantageously controlled by Government, and, if so, in what manner.

Bákhar, also known in Northern India by other names, such as *murcha* in Darjeeling, *ranu* in the Central Provinces, and *u-t-iat* in the Khasi Hills, is the artificially prepared ferment or inoculum used in N.-E. India n the manufacture of rice beer, *pachwai*, and of the rice spirit distilled therefrom. A full and interesting description of its manufacture and properties was given by J. C. Ray in a paper published in Vol. II, No. 4 of the *Journal of the Asiatic Society of Bengal*, containing an account of the manufacture and use of *bákhar* in the Cuttack District, and of the author's experimental work aimed at determining the source of fermenting power of this preparation and the conditions necessary for its successful use; the author arrives at the conclusion that saccharification of the rice starch is due to the diastasic activity of a *Mucor* introduced in the *bákhar*, to which organism he gives the name of *Mucor-Torula* and also ascribes the subsequent alcoholic fermentation to its activity. No doubt there were budding *Mucors* present in the *bákhar* which he examined, but it does not seem necessary to conclude that the alcoholic fermentation was due to their action and that yeasts were absent; in only one specimen of *bákhar* examined in this laboratory was there total absence of yeasts, and although some *Mucors* can ferment the maltose which

their diastasic action has produced from rice starch, it is much more probable that the alcohol formed by using *bákhar* is mainly produced by the fermentative power of yeasts which were present in the latter or found their way into the liquor at some stage of manufacture. It is possible, however, as has been suggested to one of us by the Chemical Examiner for Customs and Excise, that the high percentage of alcohol sometimes found in *pachwai*, amounting in some cases to as much as 22%, may be due to *Mucor* fermentation persisting after the upper limit of alcoholic concentration possible by yeasts has been reached.

Bákhar, then, is an artificial culture containing living fungi or their spores together with yeasts. The function of the former is to saccharify the rice starch and of the latter to ferment the sugars thus produced, the result being a watery solution containing dextrin, maltose, and alcohol together with smaller quantities of by-products. This watery solution in the earlier stages of manufacture may be strained off and is then known as *pachwai* or rice beer, or after further addition of water and a longer period of fermentation may be distilled to produce rice spirit.

The necessity for the presence in *bákhar* of saccharifying moulds known as " amylo ferments " arises from the essential difference between the husked rice grain and that of barley, which lies in the absence of the embryo and its associated enzyme production, from the former. Barley grain, owing to the enzymic activity of its embryo, which is not removed in husking, can be " malted " simply by keeping the grain moist and warm, when the secretion of diastase characteristic of the ensuing germination commences the conversion of starch into sugar, known as saccharification, which results in the production of " malt." Thus barley starch is converted into sugar simply by artificially inducing a natural process, whereas in the case of rice, in the process of husking, the embryo, owing to its external position, is broken away, carrying with it the self-contained power of saccharification possessed by the complete grain, and making it necessary, when alcohol production is aimed at, to ensure the presence in the ferment of some saccharifying agency.

The fermentation of rice and other food-stuffs is also carried out in other Eastern countries in a similar manner, the saccharifying power of various amylo ferments being utilized for this purpose. Examples of this use of fungi are found in the case of Saké, the Japanese rice beer, *Aspergillus Oryzæ* being the amylo ferment in this case, and also in the preparation of Soja or Bean wine and of Miso, Bean mash.

Aspergillus Batatæ = *Sterigmatocystis Batatæ* (Saito) Sacc., is used in the manufacture of yam brandy in Japan.

Aspergillus Wentii Wehmer for Soja and for Bean mash, in China.

Mucor javanicus Wehmer and *Rhizopus Oryzæ* Went for rice beer (arrack) in Java.

Mucor Rouxii (Calm.) Wehmer in Chinese yeast.

In all these cases the saccharifying power of the various ferments is utilized to change starch into sugar, generally as a preliminary to alcoholic fermentation by yeasts.

In Europe, the saccharifying power of certain fungi is extensively made use of in the amylo-process and in the commercial production of sugar from starch.

The origin of *bákhar* has no record in India, but it can be conjectured that its mode of preparation has always been the result of pure empiricism combined with observation of the conditions under which fermentation naturally occurs. As compared with the accidental discovery of barley wine, which is of such ancient date as to be popularly relegated to antediluvian times, the mode of preparation of rice beer, requiring the intervention of an amylo ferment as a preliminary to the action of yeast, is very much more complex and would require either a more fortunate series of accidents or much more power of observation on the part of the primitive man who first discovered it. It may be conjectured that accidental wetting of stored grain in the case of barley, followed by germination, and coincident with such shortness of the supply as to make its rejection as food on this account unlikely, might lead to the discovery of the increased sweetness of the grain due to saccharification of the starch, and to the further step by which the water used for cooking the grain would be found more palatable for the same reason. The accidental infection of this barley malt extract by air-borne yeasts and the resulting fermentation would inevitably follow, especially in those warm countries from which tradition derives the alcohol habit, and the same shortness of provisions which probably gave rise to the use of such other apparently spoilt articles of food or drink as sour milk, Roquefort cheese, Bombay duck (*bumla*) and vinegar, would lead to the discovery of beer.

In rice-growing countries the case would be different on account of the different structure of the rice grain, and even when the unhusked grain had been stored this would probably be for seed and would not be kept long enough to run the same risks of deterioration as the food-supply. The preliminary stage of mouldiness necessary for saccharification would no doubt occur in

the cooked or partly cooked rice put on one side for future consumption, but in most cases the resulting growth of moulds would be accompanied by such olfactory evidences of decay as to give pause to the hungriest cave dweller. The exceptions to this rule will, however, by their character explain the introduction of the use of moulds as a preliminary to fermentation. Certain fungi, notably the one, *Aspergillus Oryzæ* (Ahlbury) Cohn, in common use for brewing Saké in Japan, when grown on rice produce a pleasant flavour suggestive of apples, which is so pronounced as to make it probable that primitive man would welcome its introduction into the limited repertory of his larder instead of immediately rejecting it as unfit for human consumption. The discovery of the sweetening of the rice which accompanied the improved flavour would no doubt help to confirm this view, and it would be only a matter of time before the cooking water would be found to be palatable as a drink and its storage would lead to fermentation by air-borne yeasts.

We know little or nothing at present as to the geographical distribution of fungi, but it may be conjectured that the occurrence of such moulds as produce the abovementioned flavour was noted as being more frequent in certain localities, especially in the hills, and the same sort of observation which led to the knowledge of the properties of herbs and simples would discover in time not only the probability of securing a growth of the coveted fungus in certain localities at certain times of year, but later would associate this with habitations and finally with the vessels used for holding the mouldy rice. From this to discovery of the use of the latter as a means of producing similar growth on fresh rice would be an easy step, and may in fact have preceded the knowledge of local infection in some cases, but it seems fairly certain that we have in the existence of certain low caste tribes, who alone possess the secret or tradition of the preparation of *bákhar*, an interesting case of the handing down from one generation to another of special knowledge originating partly from accident and partly from observation, but now almost entirely lost as real knowledge of cause and effect and merely surviving as blind tradition. This is well illustrated in the case of *bákhar* by the traditional introduction into the mixture of powdered fragments of bark, roots, or leaves of various jungle plants ; the original makers of *bákhar* no doubt found that the use of certain ingredients increased the chances of growth of the desirable moulds, but in common with other holders of trade secrets they would naturally add numerous other insignificant items in order to prevent their knowledge from passing into the possession of outsiders. The probable function of some of the ingredients now used by various *bákhar* makers will be considered later in this paper but at present it may be said with confidence that the makers

themselves have no real knowledge of the specific functions of any of them, and in consequence the manufactured article varies in composition and quality from one sample to another and from place to place.

As an instance of such ignorance and its effect upon the quality of the *bákhar*, it may be mentioned that an *Aspergillus flavus* Link of comparatively high diastasic power was found associated with the leaves used by the *bákhar* makers in the Khasi Hills, and there is a strong probability that their progenitors introduced these leaves on account of their experience of the powers of this amylo ferment; the present generation, however, completely sterilize whatever vegetable tissues are added, either roots, bark or leaves, by singeing them over the fire so as to make them brittle enough to break up in the mortar and so mix readily with the powdered rice. In this way ignorance entirely eliminated what may originally have been intended to ensure the introduction of fresh amylo ferment, in case this should die out in the ordinary course of transferring from one batch to another, as no doubt frequently occurs. The present method is simply to dust the new cakes with powder from the old and so carry on the growth of the mould indefinitely. It seems probable from examination of the air-borne mould spores in the *bákhar* makers' houses in the Khasi Hills that reinfection does not entirely depend upon intentional transfers, but is almost certain to occur on account of the large number of spores of the local *bákhar* moulds, of several kinds, invariably present in the air of the houses. It may be mentioned here that cake from the same makers does not always contain the same ferments.

One fact which helps to prevent the total extinction of the industry is the possibility of rejecting the *bákhar* during manufacture and before sale or use, by using knowledge based on experience and merely involving observation of the condition of the cake within a few hours of manufacture. This prevents the sale of entirely bad *bákhar*, but does nothing to ensure its quality as being more than moderate, and in fact the spirit produced by its use may be seriously defective in quantity or quality owing to the absence of effective yeasts or the presence of deleterious moulds.

It is for these reasons that the writers have undertaken an inquiry into the constitution of *bákhar* from various sources with a general idea of determining the possibility of regulating its manufacture.

In addition to the composition of the *bákhar* with regard to efficiency due to the presence of the necessary amylo ferments and yeasts, the deliberate addition of deleterious substances intended to produce intoxication or narcosis has to be considered. According to the Report of the Excise Commission

of Bengal for 1883-84 such substances as *Datura*, *Nux vomica*, and *Cannabis* are sometimes added, no doubt with a view to securing a reputation for potency for the products of individual *bákhar* makers; these are extreme cases; others of less importance include flavouring matters and bitters, all of which can only appear in the rice beer and cannot seriously affect the spirit distilled therefrom. It is probable, however, that in some cases the beer itself might be fortified by addition of distilled spirit so that the deleterious substances contained in the *bákhar* would help to produce an extremely noxious beverage. It is natural that no information on this point has been supplied along with the samples of *bákhar* obtained for the purposes of the present inquiry, which has been confined to dealing with the organisms found in the samples and the plants which the makers in various districts of N.-E. India profess to utilize. It will, however, be obvious in view of the fact that *bákhar* in Bengal is generally made by low caste hill tribes and utilized for brewing rice beer by another entirely distinct class that the quality of the product is likely to be a source of anxiety to the latter and could well form a subject for inquiry with a view to control. It will be seen from the following account how varied the character of such a product of ancient tradition can be and how little the author of the above mentioned paper is justified in generalizing as to the microflora of *bákhar* from the particular case upon which he reported.

The principal objects of the inquiry were to ascertain :—

(1) The kinds of micro-organisms present in various samples of *bákhar*.

(2) Their relative physiological activities with reference to
  (*a*) Saccharifying power (amylo ferments).
  (*b*) Alcohol production (*Saccharomycetes*).

(3) The function of the added substances such as roots, stems, or leaves of jungle plants.

(4) The reciprocal relationships between beneficial and detrimental organisms found in the *bákhar* or likely to become intrusive during its use.

(5) The possibility of acquiring a sufficient knowledge of the subject to allow of showing the makers of *bákhar* better and more reliable methods of producing this ferment.

The Manufacture of *Bakhar*.

Bákhar is made and used in the following manner, which varies very little from one district to another and appears to be practically identical,

so far as the main principles are concerned, in all those cases which the writers have examined ; such differences as were noticed consisting in the addition of spices and condiments where *pachwai* or beer was the principal object, whereas when distilled spirit was aimed at and *pachwai* became merely a by-product, the ingredients were correspondingly reduced in number.

In Darjeeling the outer skin of the root of certain wild plants [that used by the Nepalis is known to them as *bhimsenpati* and was identified by the Curator of the Lloyd Botanic Garden as *Buddleia asiatica* Lour. whilst the Limboo tribes use *wading-hang-ma* (*Polygala arillata* Ham.)] is dried and powdered ; four ounces of this material, half an ounce of ginger, three pieces of red pepper (chillies), and eight pounds of ground rice are kneaded into a stiff paste with water, made up into small round cakes about half an inch thick and dusted over with powdered cake from a previous batch ; the cakes thus prepared are wrapped in fern leaves, placed on a mat in a dark corner of the house, and left undisturbed for three days, when they are taken out and dried, preferably in the sun, and are subsequently kept dry by hanging in a cloth above the fire place.

In the Central Provinces a preparation similar to *bákhar*, and known locally as *ranu* is utilized for brewing *pachwai ;* the following account of its manufacture is taken from the Report of the Excise Committee for Central Provinces, 1904.

" The ingredient called *ranu* is compounded of a number of roots as noted in the margin "—(here follows a list of the vernacular names of fourteen plants which were identified by the Economic Botanist to the Survey of India as including the following :—

Elephantopus scaber—Vernonia—Clerodendrum—Cleistanthus collinus, Asparagus racemosus—Mesua ferrea—and *Piper betle*).

" No spices are made use of. The said roots are pounded with a sprinkling of water and their juice extracted. The flour of *arva* rice is mixed with it and made into a paste and rolled up into balls. The balls are put on straw in a closed room for two days and two nights and on their beginning to smell like bile, they are dried in the sun."

Two points of interest occur in this description :

(1) The powdered roots, etc., are not added to the rice but only a watery extract from them.

(2) No old cake is used as an inoculum for dusting the new batch.

The first point will be referred to again when dealing with the probable function of the added roots, etc., the second point of difference from the

Bengal method is either due to an error of observation on the part of the compiler of the report, very likely to occur, or implies the presence of the necessary infecting mould and yeast spores either in the materials used or in the air of the house where manufacture was carried on. The writers' experience would lead them to favour the first alternative, as although experiments carried out in the Khasi Hills, at Mowlai near Shillong, shewed the presence both of amylo ferments and yeasts in the air of the houses in which *bákhar* manufacture was carried on, this source of infection might easily fail on so many occasions as to render it unpopular even with the most primitive empiricist, in addition to which the origin of the method, if at all in accordance with the theory put forward in the Introduction, would make such a departure from tradition highly improbable.

In the Khasi Hills the method of manufacture is very similar to those above described, differing in only one important particular: only one of two kinds of added substance seems to be used in each batch, either the leaves of what appears to be a jungle grass similar to thatch grass (*Imperata arundinacea*) or the bark or stem of a creeper; the writers were unable to obtain fresh specimens of either of these for identification. Portions of either one or other of these two materials were added to the powdered rice, but the interesting point of difference lay in the fact that before the addition the material was dried rapidly over the fire in such a way as to singe it and completely destroy any adherent spores, whether of moulds or yeasts, thus removing any chance of its action as an inoculum for the *bákhar*. In this district, however, each cake as it was made was dusted with powdered fragments of a previous batch, which would secure transmission of some at least of the organisms present in the latter.

The *bákhar* cake made in this manner is in the shape of balls or discs varying in size from that of a small walnut to that of a large watch; the colour is light biscuit or white; the black mouldy specimens described by Ray[1] would be rejected as worthless by any of the users of whom the writers have had experience, being probably contaminated with *Aspergillus niger* v. Tiegh. or some similarly unsuitable organism; such contamination is liable to occur, but shows itself during the ripening of the cakes, which is brought about in the following manner.

The cakes as soon as they are made are set to dry as above described, but before this is complete, vegetative growth of the organisms present takes place. The moulds make rapid growth, their diastasic power enabling them to

[1] *Loc. cit.*

penetrate the moistened rice grains, which after a few days are completely invested with fungal mycelium ; aerial hyphæ and conidia are produced on the surface of the cake, which is found after a few days to have swelled up and become dry, whilst the rice particles are held together by the fungal mycelium, which, on breaking the cake, can be seen penetrating the mass. The success of the batch is recognized by the completeness with which fungal growth has taken place, this being judged of by the swelling of the cake and the investiture and binding of the rice grains by mycelium ; in addition to this there must be an absence of blackening and of bad odour ; the writers were not able to obtain any definite first-hand information as to positive olfactory evidences of good quality notwithstanding the recommendation of the biliary qualification quoted from the Central Provinces report ; this is perhaps no cause for surprise if the character of the surroundings in which *bákhar* manufacture is carried on is taken into account ; the Khasias, for instance, are more addicted to the use of tobacco than of soap, nor do the domestic complications resulting from their habit of supporting pigs upon the by-products of the brewery and the intense appreciation shown by these animals of the alcoholic content of the latter, tend to simplify the olfactory complex obtaining in the *bákhar*-maker's house.

This point is of particular interest, however, in connection with any attempt to modify the manufacture of *bákhar*, if this is to be done by encouraging the use of comparatively pure cultures of amylo ferments and yeasts. Thus in the case of *Aspergillus Oryzæ*, the mould used by the Japanese for making their rice beer, Saké, the growth of this organism on rice is accompanied by the production of a decided and pleasant odour of apples, the absence of which would at once indicate probable failure of the brew. The quality of this flavour is modified by the action of the yeasts present and may afford valuable indications of the character of the fermentation likely to be obtained by use of the sample in question, but its production depends first upon the efficiency of the amylo ferments and it has been found that certain moulds such as *Aspergillus niger* and a commonly present *Dematium*, although causing the cake to rise and binding the rice grains together, failed to give the odour and produced bad fermentation. As mentioned above, the makers of *bákhar* in the Khasi Hills, who are generally women, did not make use of odour as an indication of quality ; when invited to express opinions as to the quality of samples of *bákhar* made in the Provincial laboratory at Shillong, from various materials, they condemned a sample, similar in all respects to those of their own manufacture except in the fact that no jungle leaf had been added, on account of the absence of the yellowish tinge due to this

addition. This sample, however, produced good fermentation. Another sample, although containing no yeast, was favourably received on account of its sweet taste due to saccharification by the amylo ferment present. In this case the absence of the characteristic yeast odour might have been taken as an indication of the lack of fermenting power which it actually showed upon trial. Another sample was favourably judged on account of its yellow colour, this being due to the ripening of the conidia of the *Aspergillus Oryzæ* present although the absence of yeast deprived it of fermenting power. It will be obvious from the above instances that the most expert maker of *bákhar* may be not only entirely ignorant of the necessary constituents of this material but unable to judge of its quality, and in fact the Khasi women willingly admitted that batches of apparently good *bákhar* were liable to fail, this failure being generally ascribed by them to some malign and generally immaterial influence.

The first step which would seem to suggest itself in any attempt to improve the manufacture of *bákhar*, would be to introduce the use of one amylo ferment such as *Aspergillus Oryzæ* in place of the heterogeneous mixture of moulds at present found in this ferment. Experiments to determine the feasibility of this suggestion have been carried out and a description of the results will be found later in this paper.

From the above account it will be seen that the *bákhar* maker is able to get some idea of the presence or absence of the necessary amylo ferments in his cake at an early stage in the manufacturing process, and in the same way the *pachwai* brewer can judge of this point when buying *bákhar;* no such certain indications, however, are afforded as to the presence or absence of the yeasts or *Saccharomycetes*. Notwithstanding the opinion expressed by Ray,[1] and the well-known alcohol producing power of certain moulds, the alcoholic content of *pachwai* produced by the use of *bákhar*, in all the cases examined in this laboratory, appeared to depend almost entirely upon the presence and activity of yeasts. Even if the moulds present in *bákhar* were able to produce the necessary alcoholic fermentation the amount and quality of this would vary very much, in accordance with the composition of the micro-organic content of the cake used, thus increasing the uncertainty of the action of the latter. The same argument however applies to the yeast content, as this has been found to vary, as is shown in the biological analyses of samples of *bákhar* given on page 147 and as there is no reliable method of determining the yeast content or alcohol producing power of samples of *bákhar* except by actual trial, the brewer cannot assess the value of such samples from this

[1] *Loc. cit.*

point of view. This is the most unsatisfactory feature of the present system under which the maker of the ferment and the user are distinct. As will be seen later the writers would advise the brewer to provide his own yeast, even should he find it convenient to purchase his amylo ferment in the shape of *bákhar*.

Pachwai is made by adding powdered *bákhar* to half-boiled rice in the proportion of about one of the former to one hundred of the latter; the mixture is kept in a basket, or in some localities made into cakes, and allowed to incubate for 24 hours, during which time the amylo ferments spread throughout the added rice and commence saccharification of the starch of the latter. The infected material is then transferred to earthen jars which are filled up with water; fermentation sets in and is completed in from 3 to 5 days according to the temperature. The resulting liquid is then strained off and may be drunk as *pachwai* or distilled to produce spirit.

Biological Analyses of *Bákhar* Cakes from Various Sources.

Samples of *bákhar* were received from Darjeeling, Ranchi, Purulia, Chaibassa, Balasore, Rajmahal, Dumka, Sambalpur and later from Shillong.

In order to ascertain what moulds and yeasts were present in the various samples these were plated on ammonium nitrate agar with the following results:—

1. Darjeeling .. *Rhizopus Cambodja* (*Chrzaszcz*) Vuill.
   Dematium.
   Penicillium.
   Yeast, one kind.
2. Ranchi .. *Mucor Prainii*, *Dematium*, *Aspergillus niger*. } (numerous colonies).
3. Purulia .. *Rhizopus*, *Dematium*, Yeast. } Poor growth in all cases.
4. Chaibassa .. *Mucor Prainii*.
   Dematium.
   Yeasts—Two varieties.
5. Balasore .. *Mucor Prainii*—Abundant.
   Dematium.
   Yeasts—Two varieties; numerous colonies.

6. Rajmahal .. *Mucor Prainii*—Abundant.
Rhizopus—Few.
No Yeasts.

7. Dumka .. *Mucor Prainii*—Abundant.
Dematium.
Rhizopus.
Yeast.

8. Sambalpur .. *Mucor Prainii.*
Dematium.
Yeast.

From the above analyses it will be seen that the number of kinds of moulds found in *bákhar* from geographically widely separated centres is comparatively small, arguing a very considerable efficiency in the empirical method used, so far as obtaining the presence of those moulds which we shall see possess considerable saccharifying power, and at the same time excluding more common but less desirable forms such as *Penicillium.* It will be noticed that *Mucor Prainii* Chodat and Nechitsch was found in six out of the eight samples examined ; this organism was originally isolated from *bákhar* by Nechitsch ; it is of interest to note its continued presence.

The Purulia cake gave poor growth on the agar plates ; *Mucor Prainii* was absent and the sample might be regarded as a poor one from the point of view of its efficiency as a provider of saccharifying organisms, if tested only in this way.

The Rajmahal cake was distinguished by an entire absence of yeasts and might be considered defective for this reason, although its content of saccharifying organisms was good.

The samples were also plated on ordinary nutrient agar to ascertain the presence of bacteria which would not appear on ammonium nitrate agar, being suppressed by the rapid growth of moulds which occurs on this medium.

Bacteria were found in the samples from Ranchi, Balasore and Rajmahal, but both kind and numbers were few ; this as will be shown later is due partly to the antiseptic action of the added leaf, roots, or bark and largely to the suppression of their growth by that of the moulds.

The above method of analysis cannot be considered of great value except when carried out on a very large number of samples, if it is to be regarded as a means of determining the relative efficiency of different brands of cake ; it was merely intended in this case to serve as an index of the organisms generally occurring in these cakes. The variation between samples from the same

district was also found to be considerable so far as the composition of their floral complex was concerned, but the general inference was that in the majority of samples saccharifying organisms were present, whilst there was an unexpected freedom from those more common moulds of less diastasic power, whose growth is generally accompanied by the production of by-products of offensive odour. Some samples, however, showed the presence of deleterious organisms, such as *Aspergillus niger* v. Tiegh. whilst in others the growth of appropriate moulds was feeble and insufficient to suppress that of bacteria, which rapidly produced putrefaction.

Saccharifying Power of the Various Samples.

This was determined by mixing 1 gram of powdered cake with 100 grams of rice after steaming, and keeping at 30°C. for four days, after which the sugar was extracted with water and its amount determined by Fehling solution.

The following relative amounts were obtained :—

1.	Balasore	80·6
2.	Purulia	60·9
3.	Chaibassa	58·5
4.	Ranchi	41·2
5.	Rajmahal	39·6
6.	Darjeeling	25·4
7.	Dumka	12·4
8.	*Koji*	128·4

These figures show that the first method of analysis is of little value for determining the relative saccharifying powers of samples of *bákhar*, although it might be possible to devise a modification of it which would afford more accurate information ; the direct determination of the saccharifying power is, however, more simple and at the same time gives results which are of practical value, so that ultimate analysis would probably only be useful for scientific research.

Koji, the very high figure for which shows its superiority in this respect to all the Indian preparations, is the Japanese equivalent for *bákhar*, but differs considerably from the latter in being practically a pure culture of *Aspergillus Oryzæ* (Ahlbury) Cohn made not on powdered rice, but on whole rice grain.

As will be seen from the figures given in the following experiments, made to determine the relative saccharifying power of the various amylo ferments

in pure culture, the diastasic action of these fungi is largely influenced by the proportions of air and water present, an excess of moisture being more prejudicial in the case of the *Aspergillus* than in that of the *Mucors*; in general it may be said that the technique of saccharification with amylo ferments must be carefully regulated with reference to this point, as will appear from the following results of tests of the relative saccharifying power of pure cultures.

Relative Saccharifying Power of Amylo Ferments.

Pure cultures of *Aspergillus Oryzæ*, *Mucor javanicus*, *Rhizopus Cambodja* and *Dematium Chodati* Nech.[1] were inoculated into 1% starch solution and incubated at 30°C., examining from time to time for disappearance of starch; this took place after the following number of days.

Aspergillus Oryzæ after 11 days.
Rhizopus „ 15 „
Dematium „ 20 „
Mucor javanicus:—starch still present after one month.

After eight days the amount of sugar formed was determined with Fehling. The relative amounts were:—

Aspergillus Oryzæ	23·0
Rhizopus	16·4
Dematium	14·6
Mucor javanicus	9·7

This experiment was repeated, other moulds being included. The absence of starch was noted as follows:—

Aspergillus Oryzæ after 8 days.
Aspergillus niger „ 17 „
Rhizopus Cambodja „ 17 „
Dematium „ 20 „
Mucor javanicus „ 27 „
Mucor Prainii 1 & 2, *Monilia* } Still present after 30 days.

Mucor Prainii 1 & 2.—In several cases a *Mucor* with a very characteristic dendritic growth and other morphological characters apparently distinct

1 All the fungi isolated from *bákhar* were kindly identified by the Imperial Mycologist, Dr. Butler; with reference to *Dematium* he notes as follows: "The name has been changed by Saccardo to *Cladosporium Chodati* (Nech.) Sacc. who, however, remarks that it is perhaps better considered an *Oospora*: I agree."

from those of *Mucor Prainii*, was isolated from samples of *bákhar*, and in some cases both forms were found in the same sample. It was subsequently identified by Prof. Vuillemin of Nancy as being identical with *Mucor Prainii*. although the dendritic form invariably showed different physiological characters so far as saccharification of starch was concerned, especially with regard to the condition of solubility, gelatinisation, and water content of the substratum. It is referred to in these notes as *Mucor Prainii* 2.

Saccharifying Power of the Amylo Ferments on Rice Grain.

The moulds were then inoculated into 50 grams steamed rice and kept in glass jars with covers and incubated at 30°C. for 5 days, after which the sugar formed was estimated by extraction with water and titration with Fehling.

Aspergillus niger	29·0
Mucor Prainii (2)	18·2
Mucor javanicus	16·2
Dematium	13·8
Aspergillus Oryzæ	10·0
Mucor Prainii (1)	7·7
Rhizopus Cambodja	8·0

In this experiment a decided difference in relative diastasic power was found, *Aspergillus Oryzæ* taking a lower place relatively to the *Mucors*, whilst a wide separation occurs between *M. Prainii* 1 and 2. This is evidently due to the conditions of the experiment and probably a consequence of lack of aeration and excess of water, as the aerial growth of the moulds obviously differed. The experiment was repeated using petri dishes instead of covered glass jars; the rice was sterilized in the autoclave and inoculated with pure cultures. After 4 days incubation, the rice, which had dried up completely, was extracted with water and gave the following figures with Fehling:—

Aspergillus Oryzæ	31·3
Aspergillus niger	13·5
Mucor javanicus	29·6
Rhizopus Cambodja	12·3
Dematium	7·2
Mucor Prainii (1)	9·3
Mucor Prainii (2)	2·8

The growth of the *Mucors* was evidently much restricted by loss of moisture.

The experiment was repeated, in this case the moisture content being kept up by placing the petri dishes under a bell-jar with water. Even in these circumstances the moisture seemed insufficient and after two days a few c.c. sterile water was added; after 4 days the rice was extracted with water and gave the following figures with Fehling :—

Aspergillus Oryzæ	13·8
Aspergillus niger	13·4
Mucor javanicus	15·9
Mucor Prainii (1)	12·4
Rhizopus	9·7
Mucor Prainii (2)	9·0

Lack of moisture in this case also evidently retards the growth of the *Mucors* except *Mucor javanicus* which in both these experiments compares favourably with *Aspergillus Oryzæ*.

From consideration of the above experimental results it will at once be seen that the amount and rate of saccharification produced by the use of *bákhar* will depend upon a large number of varying factors, the principal one being the presence of suitable amylo ferments in an active condition. All the samples examined contained such amylo ferments, but the experiments on saccharification showed that the efficiency of the cakes would depend largely upon arranging the conditions for their use to suit the particular ferments they contain. In Japan this difficulty is overcome, or rather avoided, by the use of *koji* which, as pointed out above, is a practically pure culture of *Aspergillus Oryzæ*, the best conditions for the action of which when once ascertained can be indefinitely reproduced. So far as amylo ferments are concerned the idea of substituting one suitable organism such as *Aspergillus Oryzæ* for the haphazard mixture found in *bákhar*, at once suggests itself. This would also help to keep out undesirable organisms, such as *Aspergillus niger*, as it has been found in this laboratory that a sufficient amount of an inoculum of a fungus such as *Aspergillus Oryzæ* under favourable conditions will result in a growth sufficiently vigorous to inhibit that of any intrusive oganisms. This will be referred to again when considering the subject of laboratory-made *bákhar*.

Relative Alcohol Production by Various Samples of *Bákhar*.

This was determined by steaming 100 gm. of rice and inoculating with about 1·5 gm. of the sample. After thorough mixing, the rice was placed in wide mouthed cotton-wool stoppered bottles and incubated at 30°C.

for 24 hours, after which 250c.c. water was added to each bottle and incubation was carried on for five days at 30° C.

150 c.c. water was then added and distillation carried out.

Cake	Sp. Gr. at 20° C.	Distillate	Proof Spirit
1. Dumka	0·961	104 c.c.	61·6 c.c.
2. Ranchi	0·965	100 c.c.	53·5 c.c.
3. Darjeeling	0·959	80 c.c.	49·5 c c.
4. Balasore	0·968	95 c.c.	46·3 c.c.

Owing to the very large amount of time and labour involved in the isolation and maintenance of pure cultures of yeasts, it was not found possible to make biological analyses of the above samples from this point of view, although any research carried out with the intention of putting alcoholic fermentation in India upon a sound basis must inevitably depend for its success upon the examination of all the yeasts available in order to miss no opportunity of obtaining the most efficient forms for various substrata.

Four distinct forms of *Saccharomyces cerevisiæ* have been isolated in this laboratory, from *bákhar* (Khasi), molasses wash, beer wort and *mahua* flower (*Bassia latifolia*) respectively, and experimental work has been carried out to determine their fermenting power under various conditions. It will be sufficient to say here that the yeast from *bákhar* was the one of these four found most capable of alcohol production from saccharified rice. There is every probability, however, that research would discover more efficient strains, but it seems doubtful whether this would be worth while in the interests of the present class of consumers of *pachwai*.

Laboratory-made *Bakhar*.

A large number of experiments was made in Shillong and continued in this laboratory with a view to determining the practicability of producing *bákhar* containing pure cultures of amylo ferments and yeasts. The experiments at Shillong had the advantage of being carried out in a *pachwai*-making district, the Khasi women being quite willing to test the practical efficacy of the laboratory-made *bákhar* by their own methods under supervision. Subsequent experience at Pusa has led to several modifications of the method of manufacture, which it is hoped to test on a practical scale.

Experiments at Shillong.

Biological Analysis of Khasi Bákhar.—Local cake was plated in ammonium nitrate agar in two dilutions ; A higher and B lower dilution.

A.—*Mucor Prainii* 2.
—*Rhizopus* (Black sporangia).
—*Aspergillus* (White sporangia).
B.—*Mucor Prainii* 2.
—*Dematium* (numerous).
—Yeasts (two varieties).

This plating illustrates the necessity for extensive duplication in such analyses, just as previously cited instances show the importance of using various media in order to obtain any complete notion of the biologic content of such material. In the lower dilution B the rapidly growing *Dematium*, numerous colonies of which were present, probably suppressed the growth of the *Rhizopus* and *Aspergillus*, which were subsequently found to be almost constant constituents of the Khasi *bákhar* from this particular locality (Mowlai ; Shillong).

Effect of Inoculation with Old Cake.

The final act in the preparation of *bákhar* by the Khasias is the dusting of the new cakes with powdered fragments of a previous batch ; this inoculation serves to carry on the culture of moulds and yeasts, more or less completely, from one batch to the next ; in order to determine whether such inoculation is necessary, a *bákhar* maker was persuaded to prepare a batch of cakes omitting this dusting in the first half of it. The cakes thus made were examined by plating, when it was found that although growth took place rather more rapidly in the case of the dusted specimens, the undusted ones subsequently developed an almost identical flora ; this would seem to show that, as might have been expected, the house in which the materials were stored and the pounding of the rice carried out, was thoroughly infected with mould spores of the kinds found in the cake.

An interesting indication of some slight recognition of the basic principles underlying the process was afforded by the custom of carrying on the mixing of the dough and kneading of the cakes in the open air at some distance from the house itself, suggesting an attempt to limit infection to some extent to that introduced intentionally with the old cake ; an attempt was made to discover deliberate intention in this particular cake as some stress was laid upon it by the makers themselves, but the latter were unable to give anything more

definite than a mythological reason coupled mysteriously with missionary effort in the district, for this item in their technique.

Effect of Addition of Jungle Plants.

When this subject first came under consideration it seemed possible that the use of jungle plants might be connected with the introduction of mould spores naturally associated with them, and this view may still be held in the writers' opinion for reasons already set forth, which are briefly, that when once mouldiness had been recognized as a prime characteristic of *bákhar*, the common occurrence of moulds upon the bark and leaves of jungle plants would naturally lead to their use as ingredients of the ferment. In the case of Khasi *bákhar*, however, this does not hold good as the makers singe the leaves or bark in the fire before use, thus destroying any adherent fungal spores or mycelium.

An experiment was made to obtain an idea as to whether the addition of leaf was essential in actual practice.

Local *pachwai* makers were invited to make use of

(*a*) Local *bákhar* cake.

(*b*) Local *bákhar* cake made without leaf. This cake, made in the same manner as Khasi *bákhar*, but without the addition of leaf, on incubation developed mycelial growth similar to that of the normally prepared samples.

(*c*) Cake containing only a pure culture of *Aspergillus Oryzæ ;* this last cake was added to the experiment in order to obtain some preliminary idea as to the saccharifying power of *Aspergillus Oryzæ* on a practical scale under Indian conditions ; at the same time it was shown that under the conditions of the experiment the addition of yeast was necessary, local air-borne individuals apparently not being present in sufficient quantity in the air of the laboratory to produce vigorous alcoholic fermentation.

The use of these three cakes gave the following results :—

(*a*) and (*b*). No obvious difference in the character of fermentation except that (*b*) produced a more pleasant flavour.

(*c*) No alcoholic odour nor evolution of gas ; the liquid was distinctly sweet to the taste whereas (*a*) and (*b*) were slightly sour.

After four days 10 c.c. of the liquid was pipetted off and the relative acidity of the three samples determined by titration with $\frac{N}{10}$ soda and phenolphthalein.

(*c*) required 0·5 c.c. $\frac{N}{10}$ NaOH for neutralization.

(*b*) ,, 1·0 c.c. ,, ,, ,, ,,

(*a*) ,, 1·5 c.c. ,, ,, ,, ,,

After 6 days, distillation was carried out ; 1,500 c.c. water was added to the $2\frac{1}{2}$ litres originally used for each sample ; 700 c.c. of distillate was collected from each and the specific gravity determined at 21·5° C.

(*a*) 162·9 c.c. Proof Spirit.
(*b*) 226·7 c.c. ,, ,,
(*c*) 67·5 c.c. ,, ,,

So far as this experiment was concerned the addition of leaf had no favourable influence upon either the quantity or quality of the alcoholic fermentation.

The Khasi women who carried out the making of the *bákhar* and the preparation of the rice, prophesied the failure of the brew from the cake without leaf and probably suspected some surreptitious addition as responsible for the actual result. On the other hand, they were favourably impressed with the sugar content of the liquor containing *Aspergillus Oryzæ*, but did not expect any good result from distillation owing to the obvious absence of fermentation in this case ; they had no hesitation in pronouncing the flavour of the distillate from (*b*) as superior to that from (*a*).

A further experiment was made in which three cakes were compared in order to determine the suitability of *Aspergillus Oryzæ* as an amylo ferment in conjunction with an Indian yeast, and at the same time to test further the necessity for adding jungle plants to the cake.

Cakes were made with leaf and inoculated as follows :—

(*a*) Inoculated with Khasi *bákhar*.

(*b*) Inoculated with *Aspergillus Oryzæ* and a yeast (*D :* isolated from Khasi *bákhar*).

(*c*) No leaf added—inoculated as (*b*).

Fermentation was carried out at 30° C. for five days ; distillation gave the following results :—

(*a*) 48·8 c.c. Proof Spirit.
(*b*) 43·9 c.c. ,, ,,
(*c*) 48·0 c.c. ,, ,,

Hence (1) the addition of leaf in this case gave no positive result.

(2) *Aspergillus Oryzæ* together with local yeast gave as good fermentation as that produced by the indigenous flora.

It may be mentioned here that further experiment showed that fermentation results depend very largely not only upon the organisms present, but also upon the conditions of aeration and water content, under which their action takes place. This is more especially the case so far as saccharification is concerned and will be referred to more fully later in this paper

Function of Materials, such as, Leaves, Bark, and Spices.

In order to obtain some idea as to the function of the added materials, several experiments were carried out. These were aimed at discovering whether such additions could be useful in the following directions:—

(1) *Inocula.*—As possible conveyers of amylo ferments or *Saccharomycetes.*

(2) *Nutrients.*—As supplying substances not found in the rice, for the better nutrition of the desirable fermenting organisms.

(3) *Antiseptics.*—As possibly possessing antibacterial and preservative properties.

(4) *Flavouring.*—As modifying the flavour of the *pachwai* either directly or by influencing the character of the fermentation.

The very large number of different materials, one list of which has been given[1], used by *bákhar* makers made it probable not only that these would exercise varying functions, but that the manufacturers themselves would be ignorant both of their original intention and of their present utility. It was quite impossible to examine more than a few of these substances, but it was clear both from experiment and local inquiries that this view of their utilization was a correct one. It may be said at once that no definite conclusion could be arrived at as to the necessity for the use of any of these materials, the only positive indication obtained being that the initial growth of yeasts was favourably affected by the addition of leaves of the jungle grass used by the Khasias.

1 The Report of the Bengal Excise Commission, 1883-84, gives much detailed information as to the numerous ingredients made use of in connection with the manufacture of distilled spirit, but on the subject of *bákhar* and *pachwai* is less definite than the Report of the Central Provinces Excise Committee already referred to. It seems very probable that inquiry into this subject is likely to elicit somewhat unreliable information owing to the readiness of local authorities to give the names of all the indigenous substances used in connection with fermentation, irrespective of distinction between those actually introduced into *bákhar*, and others either added to *pachwai* during fermentation or afterwards, or used only in connection with the manufacture of distilled spirit. The writers have therefore confined their attention to those substances commonly used either by the Nepalese or Limboo tribes or by the Khasi women in Shillong, or to such as were actually contained in samples of *bákhar* received from various sources.

Frequent suggestions have been made as to the use of toxic ingredients in *bákhar* such as *Datura* and *Nux vomica*. It is possible that such substances may be added to *pachwai* at some stage of manufacture, but the following calculation seems to preclude the probability of such addition to the *bákhar* itself.

Quantities of materials used by Khasias in making *pachwai* from *bákhar* cake.

Bákhar cake	20 gm.
Rice	1,200 „
Water	2,500 c.c.

=0·80% *bákhar* cake in *pachwai* liquor.

Bákhar cake contains

Powdered leaf, etc.	4 oz.
„ rice	8 lb.

=about 3·0% leaf in *bákhar*=0·024% in *pachwai*.

Leaf or other material may contain maximum of say 5% toxic alkaloid.

Then 0·8% × 3·0% × 5%

=0·0012% alkaloid in *pachwai* liquor.

Similarly the introduction of bitter principles or other flavouring matters into *pachwai* is not likely to be effected through the *bákhar*, so far as any samples examined by the writers are concerned, as in all of them the proportion of materials added to the rice was obviously insufficient to affect the flavour of the *pachwai* by introduction into the latter in such small proportion. It would appear from the above calculation and also from experiment that the added substances can only carry out their specific function or functions within the sphere of the *bákhar* cake itself or at most only indirectly in the subsequently added rice and water, by affecting the character of the fermentation.

(1) *Inocula.*—The roots of a specimen of the plant (*Polygala arillata*) obtained from Darjeeling *bákhar* makers, when kept moist developed a fungal growth which was plated in ammonium nitrate agar and gave a pure culture identified by the Imperial Mycologist as *Aspergillus flavus* Link. This fungus however did not appear on any of the plates made from Darjeeling *bákhar* which supports the view that the *bákhar* makers in that district sterilize the added materials by flaming as is done in the Khasi Hills, not with the intention of destroying adherent fungi but in order to produce brittleness and consequent ease in comminution. On the other hand there is an equal probability that some idea of eliminating undesirable moulds or bacteria originally existed in connection with this process, as seems to be suggested not only by the

flaming of the added leaves but also by the custom of smoking the interior of some of the vessels used in this as in other industries such as the making of date sugar and *dahi*, but so far as the writers were able to ascertain, the *bákhar* manufacturer is of too primitive a type to concern himself with reasons for such details of technique. It seems probable from this and other similar experiments that the amylo and other ferments contained in the *bákhar* cake are conveyed into it partly by inoculation by dusting with an earlier batch, and partly by air-borne infection. A series of observations of the air-borne organisms in a *bákhar* maker's house throughout the year would be of interest as elucidating this point. One experiment made at Mowlai (Shillong) on 23rd October 1914 consisted in exposing an ammonium nitrate agar plate in a *bákhar* maker's house for five minutes; this plate on incubation at 30° C. gave the following organisms:—

Mucor Prainii (2) after 24 hours; two colonies; no other growth.
After one week other growths appeared:—

Dematium.
Rhizopus.
Penicillium.
Yeast (one kind).

From this experiment it would appear that the greater number of the kinds of amylo ferments generally found in Khasi *bákhar* were present in the air of the *bákhar* maker's house, so that, even if inoculation by transfer from one batch to the next were omitted, some of the necessary moulds would probably appear in the cake; this alternative source of infection depends for its practical utility upon the comparatively large number of kinds of effective amylo ferments, but might easily fail in any particular case in which a deleterious intruder capable of producing bad flavour should happen to exist in large proportion, thus in the house in which this experiment was made, the *Penicillium* might very easily have existed in such quantity as to have ousted the *Mucor Prainii* which actually appeared as the predominant organism on the plate, and had this been the case its presence would have destroyed the value of any cake depending only upon casual infection.

It may be noted that only one kind of yeast appeared on the exposed plate, whereas two or three kinds at least were generally found in the cakes themselves from this locality; this however could readily be accounted for either by reason of transfer from one batch to the next, or by the fact above mentioned that the later stages of making of the cakes at Mowlai took place out of doors; other sources of infection naturally existed in the mats on

which the cakes were placed for drying and the cloth used for covering them. In the Darjeeling district, as already described, the fern leaves used for wrapping the cakes would probably introduce various organisms.

Experiments with other samples of leaf, roots and bark did not afford any conclusive evidence as to their probable utility as inocula, nor, in view of the custom of transfer by dusting new cakes with old ones, does it appear probable that any further inquiry in this direction would be of value in this connection.

(2) *Nutrients.*—Ray[1] suggests that the probable function of the added materials is to increase the growth of the moulds owing to the introduction of additional nutrients which do not occur in the rice. Much might be said in favour of this supposition, especially as mycologists are at present not fully aware of the food requirements of fungi, and the empirical nature of many culture media in use is evident. So far as the amylo ferments are concerned, however, it may be pointed out that the object of the *bákhar* maker should be to encourage the growth of such moulds as are best adapted for the saccharification of rice starch and in the necessary absence of reliable technique which might help to keep out other intrusive organisms, the use of rice alone as a nutrient, together with the smallest number of other kinds of ingredients absolutely necessary for growth is evidently indicated. Any additions to the medium, which would allow the growth of other possibly deleterious ferments incapable of flourishing on rice alone, would therefore constitute a departure from the best practice, although it would be one which might commonly result from observation of the comparatively high rate at which moulds would grow in consequence of such addition.

The case of the yeasts appears to require consideration from a different point of view; their growth in the cake itself would probably be negligible if it depended upon the previous diastasic activity of the amylo ferments; and would not become extensive until the cake was actually in use. At this point, however, competition may begin between the *Saccharomycetes* and bacteria for the sugar produced by the amylo ferments, and the favourable result of this competition will depend upon the rate of growth and consequent fermentative activity of the yeasts. It is therefore important that the latter should obtain a good start, and the following experiment suggests that the added materials may affect the issue by providing such additional nutrients as suffice to produce this desirable effect, beginning probably in the cake itself during the early moist stage immediately after manufacture,

[1] *Loc. cit.*

and thus ensuring the presence of a considerable number of active yeast cells ready to take advantage of the sugar formed from the rice by the amylo ferments.

Experiment to determine Effect of added Material upon Growth of Yeast.

Two samples of cake were made in the laboratory :—

(1) With added leaf (from Khasi Hills).

(2) Without such addition.

Both were inoculated with a mixture of *Aspergillus Oryzæ* and Khasi cake (containing Yeast D).

This cake was incubated at 30° C. in petri dishes and allowed to dry for 48 hours, after which drying was completed in the sun. The cakes were then plated in ammonium nitrate agar and incubated at 30° C. After 24 hours incubation numerous yeast colonies and some *Aspergillus* appeared on No. 1 (leaf added). No growth on No. 2.

After 48 hours a few yeast colonies appeared on No. 2.

The photograph (Plate II) shows the two plates after four days growth. It will be noted that the added materials have not only increased the number of yeast cells, but also their vitality, so that the colonies are not only relatively more numerous but larger.

It may be stated here that this result is the only indubitably positive one obtained as the result of the addition of leaf or roots to the cake. It should be noted that such addition should not be considered as essential to success, but as increasing the probability of freedom from bacterial invasion ; thus in the Shillong experiment described previously the addition of leaf had no positive effect upon the fermentation, which would seem to imply that under the conditions of manufacture bacterial invasion does not necessarily occur but as the experience of the makers shows, is more likely to do so in the absence of added leaf in the *bákhar* cake.

As pointed out above, the added material is not likely, owing to its small quantity, to act as a nutrient in the fermenting liquor, so that its effect upon the yeasts as well as upon the moulds is probably confined to the cake itself. The food requirements of yeasts in such fermenting liquor as *pachwai* would form an interesting study which should also have practical importance ; it may be suggested for instance that the well-known phosphate requirements of yeast should be taken into account in selecting rice for fermentation, it

having been shown in connection with the study of " beri-beri " that polished rice is deficient in this necessary constituent.

It was found in an experiment at Shillong that when only an amylo ferment was present, in this case *Aspergillus Oryzæ*, no yeast being added to the mixture of rice and water, bacterial action rapidly set in, and although under certain artificial conditions the growth of bacteria is completely suppressed by that of moulds, further experiments seem to indicate that yeasts and moulds together are very much more capable of effecting this suppression. The phenomena of mutual antipathy would appear of high importance in connection with the study of fermentation, as there can be no doubt that the successful exclusion of bacteria from fermenting materials must depend almost entirely upon securing an early preponderance of the desired amylo and saccharo ferments of such a degree as to exclude the possibility of serious invasion by bacteria. Space does not permit discussion of the probable mechanism of interference, but it seems quite clear that this depends largely upon specific toxic excretions as well as upon relative rates of growth.

Antiseptics.

Six plates were made on ordinary nutrient agar, three of increasing dilution from Shillong *bákhar* cake and three in which the jungle leaf used by the Khasias in Shillong was introduced also in increasing dilutions but without cake.

Bákhar Cake.

After 24 hours incubation at 30° C.

Plate (1) *Rhizopus* growth over whole plate.
(2) *Rhizopus* and bacterial colonies.
(3) Two moulds and bacterial colonies.

Leaves only.

(1) *Rhizopus*—Agar coloured brown in vicinity of fragments of leaf. No bacteria.
(2) *Rhizopus*—No bacteria.
(3) No fungus and no bacteria.

Here we have indications of anti-bacterial action on the part of the leaf, but as pointed out above the amount of any such antiseptic present in the cake would be too small to produce any considerable effect in the *pachwai* although it might possibly act as a preservative in the cake itself.

Further experiments showed that bacteria were present in various cakes examined, but that the numbers appearing on agar plates depended upon several conditions.

(1) Whether ordinary nutrient agar or ammonium nitrate agar was used, the former allowing of bacterial growth whilst on the latter the comparatively rapid growth of moulds tended to suppress this altogether. Another experiment showed that dextrose agar exercised a similar influence to that of ammonium nitrate agar in favouring the suppression of bacteria in competition with fungi.

(2) When large quantities of cake were used as inocula more moulds and fewer bacterial colonies appeared; this would lead to the conclusion that the included leaf had an anti-bacterial effect but that this was limited by the small quantity present in the cake and was lost on dilution. Thus in further experiments in which *bákhar* agar medium was used, prepared by adding 2% agar to water extract of *bákhar* cake, no anti-bacterial effect was observed when 2 gm. cake were extracted either with hot or cold water. On the other hand the addition of 1 c.c. of extract of leaf made by shaking 2 gm. leaf in 50 c.c. water and concentrating by boiling to 20 c.c., produced inhibition of bacterial growth. A further experiment in which the above leaf extract was diluted four times, inoculated with a mixed bacterial and fungal culture obtained from *bákhar*, incubated for 4 days and finally plated in nutrient agar, gave very few bacterial colonies but numerous fungi. Several other experiments were carried out on the above lines using leaf extracts of varying concentration made both in cold and in boiling water; the results pointed to the conclusion that such extracts possessed anti-bacterial properties, but that the quantities of leaf present in the cake were generally insufficient to prevent bacterial growth in the moist rice to which the cake is added and still less in the *pachwai* itself, any such inhibitive effect as might occur being probably due to the growth of moulds and yeasts.

Flavouring.—No flavour can be imparted directly to the *pachwai* owing to the small quantity of materials added to the *bákhar* which might produce this result. Indirectly, flavour might be affected by alteration in the character of the fermentation, as seems to occur in the manufacture of ginger beer, but no experimental work was done to determine this.

Laboratory Preparation of Ferments.

A large number of experiments was carried out during the year to determine the feasibility of preparing *bákhar* synthetically with pure cultures and to ascertain the best method of doing so.

It is not proposed to give a detailed account of these experiments here, on account of their number and complexity, but a description of those illustrating the methods adopted, the positive and negative results achieved, and the conclusions arrived at, may be of interest. The original object of the investigation being kept in mind, it was desired to discover whether a ferment, either in the form of *bákhar* cake or in some equally handy condition, could be prepared from a single amylo ferment combined with a single yeast. In order to carry out this line of inquiry thoroughly it would be necessary to compare the relative efficiencies of combinations of a large number of such amylo ferments and yeasts, both singly and in combination ; this would involve whole-time work for an investigator for a considerable period, for which it was not found possible to provide in this laboratory, although the research cannot be considered complete until this has been done. In the meantime two organisms were selected, *Aspergillus Oryzæ*, and a yeast (*S. cerevisiæ* D) isolated from Khasi *bákhar*. A few experiments were made using other amylo ferments and yeasts from molasses wash, beer wort and *mahua*, but no results as good as those from *Aspergillus Oryzæ* and the Yeast D were obtained, although there were indications of possible improvement by the use of more than one amylo ferment at a time and this is a line of inquiry which should be followed up.

As might have been expected it was found very early in the inquiry that the method of preparing the rice for fermentation was of prime importance : thus the addition of water to the dry rice so as to produce a favourable condition for fermentation could only be done with success in one way ; the rice is thoroughly washed with cold water and then heated carefully up to boiling point with just sufficient water to allow of complete absorption of the latter by the rice, which swells up but does not become sticky or gelatinous, as occurs if either the preliminary washing with cold water or careful measurement of the amount added before heating is omitted. This is in fact the proper method of cooking rice and is no doubt well known to natives throughout India although by no means familiar to the English cook. In the laboratory the boiling temperature was arrived at by heating in the Koch sterilizer at 100° C. The Khasi women heat the rice in an open brass vessel directly over the fire, but avoid high temperature and burning by stirring the rice and towards the end of the operation by lessening the heat of the fire.

Similarly the amounts of water used in making up the cake must be carefully regulated, and it appears necessary to vary them in accordance with the amylo ferment present; thus the *Aspergilli* will not flourish on a high moisture content such as favours the growth of the *Mucors*, and in a cake made with a mixture of fungi the amount of water present will materially affect its biological character.

The following table shows the alcohol-producing power of ferments prepared in various ways in the laboratory:—

Table showing the amount of alcohol produced from 100 *gm. rice inoculated with various ferments.**

Inoculum	Sp. Gr. at 20° C.	Distillate c.c.	Alcohol % by volume	Proof Spirit c.c.
(1) Khasi *Bákhar*, Shillong	0·970	100	25·9	45·6
(2) *Bákhar* cake made in the laboratory at Shillong dusted with some Khasi cakes. (Leaves, etc., added) ...	0·974	110	22·1	42·8
(3) *Aspergillus Oryzæ* + Yeast D. Kept sterile (11 days)	0·980	120	16·1	34·1
(4) *Aspergillus Oryzæ* + Yeast D. Not kept sterile (11 days)	0·987	125	9·7	21·2
(5) *Mucor* and *Aspergillus* obtained from the Shillong cakes + Yeast D. ...	0·978	110	18·1	35·2
(6) Khasi *Bákhar*, Shillong (5 days) ...	0·9703	100	25·6	45·1
(7) Culture of *Aspergillus Oryzæ* and Yeast D. on steamed rice; incubated at 37° C (4 days)	0·983	120	13·2	28·2
(8) *Bákhar* cake prepared with the culture above (5 days)	0·9758	100	20·3	35·8
(9) *Bákhar* cake made with a culture of *Aspergillus Oryzæ* and *Sacc. cerevisiæ* (Beer) 5 days	0·9759	100	20·2	35 6
(10) *Bákhar* cake made with a culture of *Aspergillus Oryzæ* and *Sacc. cerevisiæ* (Beer) + an emulsion of *Dematium Chodati*	0·9790	112	17·1	33·7
(11) *Bákhar* cake prepared with *Aspergillus Oryzæ* + Yeast D. + *Mucor Prainii* (2)	0·9685	100	27·3	48·0
(12) *Bákhar* cake prepared with the addition of leaf and dusted with some of the Khasi cake	0·9680	100	27·8	48·8

* Unless otherwise stated, all these were incubated at a temperature of 30° C., and the alcohol distilled off 6 days after the inoculation.

Table showing the amount of alcohol produced from 100 *gm. rice inoculated with various ferments*—contd.

Inoculum	Sp. Gr. at 20° C.	Distilate c.c.	Alcohol % by volume	Proof Spirit c.c.
(13) *Bákhar* cake prepared with *Aspergillus Oryzæ* + Yeast D. to which some leaf (Khasi) had been added.	0·971	100	25·0	43·9
(14) Rice grain moistened with nutrient solution and steamed; inoculated with *Aspergillus Oryzæ*. After the fungus had grown, dried in the sun, powdered, moistened with an emulsion of Yeast D. made into cake and incubated (4 days)	0·9895	100	7·6	13·3
(15) Rice powder moistened with sterile water and inoculated with *Aspergillus Oryzæ*. After 40 hours, added an emulsion of Yeast D. and made up into cakes. Incubated at 30° C (5 days) (Cake K)	0·968	100	27·7	48·8
(16) After the growth of the *Aspergillus* on the rice powder an emulsion of Yeast D. was added and well mixed. No cakes were prepared with this. Kept in a Petri dish at 30° C; after 24 hours transferred to 37° C and the dish kept open. (K_1)	Poor fungus growth; bacterial contamination and bad odour. While distilling, charred and was rejected.			
17) Repeated with No. 15 again to see whether fermenting power is impaired by storage (4 days) (K) ...	0·990	100	7·2	12·6
(18) Cake prepared in the same way as No. 15. Rice powder moistened with sterile water, seeded with *Aspergillus Oryzæ*, after growth of fungus, well powdered and cakes made with an emulsion of Yeast D. (K_2)	Bacterial contamination and putrefactive smell.			
(19) Repeated with (K_2)	0·972	100	24·0	42·2
(20) Inoculated steamed rice with a dried culture of *Aspergillus Oryzæ* on steamed rice, 66 days old; incubated for 24 hours and then inoculated with cake K_2:—incubated at 30° C for 24 hours and then added water. (5 days)	0·965	102	30·3	54·6
(21) Repeated No. 20. (5 days)	0·965	110	30·3	58·9

It will be seen that under the conditions of these experiments considerable differences in fermenting power are exhibited by variously prepared ferments, and although no experiments on a large scale, excepting those at Shillong already described, have been carried out, there is good reason to suppose that similar variations would occur on a commercial scale. It is unfortunate that in the absence of an expert it was impossible to report upon differences of

taste and smell, as these were shown to a considerable degree and would no doubt seriously affect the choice of organisms for use. The characteristic and pleasant apple odour associated with the growth of *Aspergillus Oryzæ* would lead the amateur to suppose this a suitable organism for use in the production of potable liquor, but it is of course very possible that the educated taste of the consumer of *pachwai* might lie in some other direction.

(11) The high figure obtained in this experiment suggests the possibly greater efficiency of a compound as compared with a simple amylo ferment.

(14) Here a complete mineral nutrient solution containing phosphates, potash, lime, magnesia and iron was added in order to ascertain whether any of these elements might be a limiting factor in the growth either of the fungus or the yeast. The resulting low figure no doubt indicates the interference with the normal fermentative processes of the yeast by the excess of nutrients introduced.

(15) This experiment and the resulting Cake K was the first attempt to produce *bákhar* in two separate stages, the first one being intended to secure a good growth of the amylo ferment with an accompanying supply of sugar, the second one introducing the yeast the development of which will be facilitated by the presence of this supply.

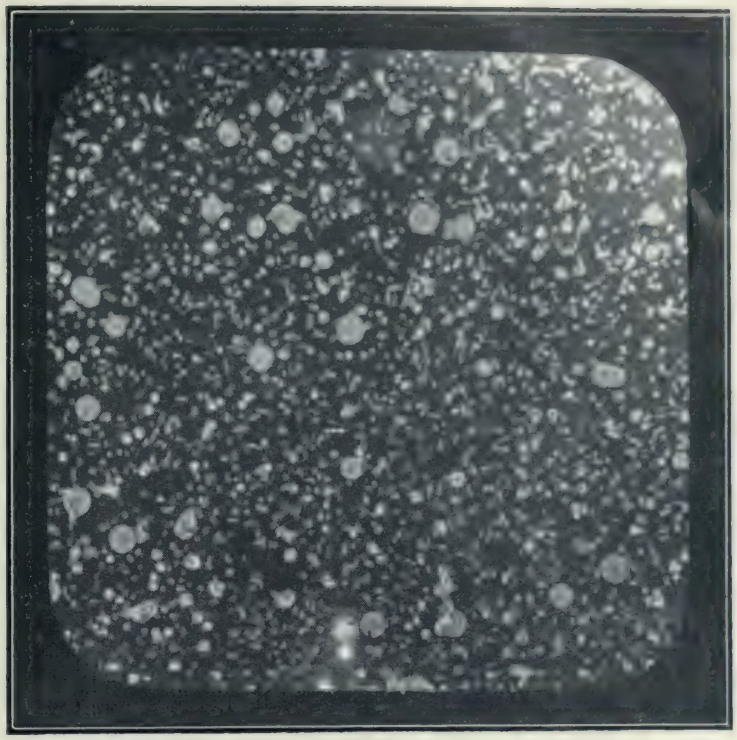
(16 & 18) In these experiments, bacterial contamination occurred resulting not only in putrefactive odours but in complete absence of fermentation. It was noted that where this had occurred distillation was characterized by charring and excessive frothing due to the presence of bacterial mucilage. It is not possible at present to say why bacterial infection should have occurred in these two cases as it appears to do occasionally in actual practice, as in experiment 19, successful fermentation was obtained with the same inoculum. It is clear however that failure in growth of the amylo and saccharo ferments must almost inevitably result in such bacterial invasion, which is only prevented by the preponderance of the fungal flora.

(20) In this case and in 21 which was a repetition of the same method, an imitation of the Japanese Koji (*Aspergillus Oryzæ* on steamed rice) was prepared and used to inoculate the steamed rice, which, after 24 hours of its saccharifying action, was then further inoculated with a cake (K_2) containing Yeast D, and again incubated for 24 hours, after which the requisite amount of water was added and fermentation kept up for 3 days. This method gave higher yields of alcohol than any previously adopted and its success in this respect suggests its trial on a practical scale. Further than this the writers would recommend the adoption of the following modification of the usual

process, namely, that in order to secure more certain development of a good type of yeast the brewer should not depend upon the *bákhar* for this constituent, but should make use of active wash from his own vats to inoculate the rice which has gone through the preliminary stage of saccharification above described, or failing this should obtain a separate supply of yeast from a reliable source. This seems to be the logical conclusion drawn from the above described experiments which appear to indicate that variations in quality of the *bákhar* accompanied by fermentation failures are not so much to be attributed to absence of efficient amylo ferments as to that of good types of *Saccharomycetes*. It also appears probable that better results would be obtained if the diastase secreted by the *Aspergillus* were destroyed by heating before the addition of the yeast, the action of which is seriously interfered with by the presence of any considerable proportion of this enzyme in the sugar solution to be fermented. The multiplication and activity of the yeast would also be increased by aeration of the liquid. It must be emphasized, however, that this inquiry, although involving a large amount of work, is by no means complete in any direction, as has been pointed out in the course of the above description of the methods employed in attacking the problems presented. In particular, the question of flavour although apparently of vital importance from a commercial point of view, has necessarily been left almost entirely out of consideration.

PUSA,
April 1915.

ASPERGILLUS ORYZAE
on Ammonium nitrate agar
one week old.



BÁKHAR YEAST 28-4-15. WITH ADDED LEAF.

BÁKHAR YEAST 28-4-15. NO LEAF.

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